

ACETYL-COENZYME A CARBOXYLASE 2 AS A TARGET IN THE  
REGULATION OF FAT BURNING, FAT ACCUMULATION,  
ENERGY HOMEOSTASIS AND INSULIN ACTION

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This non-provisional patent application is a continuation in part of, claims benefit of, U.S. Serial No. 09/749,109, filed December 26, 2000.

Federal Funding Legend

This invention was produced in part using funds from the Federal government under N.I.H. G.M. 19091. Accordingly, the Federal government has certain rights in this invention.

## Field of the Invention

The present invention relates generally to the field of fat metabolism and weight control. More specifically, the present invention relates to the role of the ACC2 isoform of acetyl-CoA carboxylase in regulating fatty acid accumulation and oxidation.

## Description of the Related Art

Acetyl-CoA carboxylase (ACC), a biotin-containing enzyme, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, an intermediate metabolite that plays a pivotal role in the regulation of fatty acid metabolism. It has been found that malonyl-CoA is a negative regulator of carnitine palmitoyltransferase I (CPTI, a component of the fatty-acid shuttle system that is involved in the mitochondrial oxidation of long-chain fatty acids. This finding provides an important link between two opposed pathways—fatty-acid synthesis and fatty-acid oxidation. Thus, it is possible to interrelate fatty acid metabolism with carbohydrate metabolism through the shared intermediate acetyl-CoA, the product of pyruvate dehydrogenase. Consequently, the roles of malonyl-CoA in energy metabolism in lipogenic (liver and adipose) and non-lipogenic (heart and muscle) tissues has become the focus of many studies.

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In prokaryotes, acetyl-CoA carboxylase is composed of three distinct proteins—the biotin carboxyl carrier protein, the biotin carboxylase, and the transcarboxylase. In eukaryotes, however, these activities are contained within a single multifunctional protein  
5 that is encoded by a single gene.

In animals, including humans, there are two isoforms of acetyl-CoA carboxylase expressed in most cells, ACC1 ( $M_r$  ~265,000) and ACC2 ( $M_r$  ~280,000), which are encoded by two separate genes and display distinct tissue distribution. Both ACC1 and ACC2 produce  
10 malonyl-CoA, which is the donor of the “C<sub>2</sub>-units” for fatty acid synthesis and the regulator of the carnitine palmitoyl-CoA shuttle system that is involved in the mitochondrial oxidation of long-chain fatty acids. Hence, acetyl-CoA carboxylase links fatty acid synthesis and fatty acid oxidation and relates them with glucose utilization and  
15 energy production, because acetyl-CoA, the substrate of the carboxylases, is the product of pyruvate dehydrogenase. This observation, together with the finding that ACC1 is highly expressed in lipogenic tissues such as liver and adipose and that ACC2 is predominantly expressed in heart and skeletal muscle, opened up a  
20 new vista in comparative studies of energy metabolism in lipogenic and fatty acid-oxidizing tissues.

Diet, especially a fat-free one, induces the synthesis of ACC's and increases their activities. Starvation or diabetes mellitus represses the expression of the *Acc* genes and decreases the activities of the enzymes. Earlier studies addressed the overall activities of the carboxylases with specific differentiation between ACC1 and ACC2. Studies on animal carboxylases showed that these enzymes are under long-term control at the transcriptional and translational levels and short-term regulation by phosphorylation/dephosphorylation of targeted Ser residues and by allosteric modifications induced by citrate or palmitoyl CoA.

Several kinases have been found to phosphorylate both carboxylases and to reduce their activities. In response to dietary glucose, insulin activates the carboxylases through their dephosphorylation. Starvation and/or stress lead to increased glycogen and epinephrine levels that inactivate the carboxylases through phosphorylation. Experiments with rats undergoing exercises showed that their malonyl-CoA and ACC activities in skeletal muscle decrease as a function of exercise intensity thereby favoring fatty acid oxidation. These changes are associated with an increase in AMP-kinase activity. The AMP-activated protein kinase (AMPK) is activated by a high level of AMP concurrent with a low

level of ATP through mechanism involving allosteric regulation and phosphorylation by protein kinase (AMP kinase) in a cascade that is activated by exercise and cellular stressors that deplete ATP. Through these mechanisms, when metabolic fuel is low and ATP is  
5 needed, both ACC activities are turned off by phosphorylation, resulting in low malonyl-CoA levels that lead to increase synthesis of ATP through increased fatty acid oxidation and decreased consumption of ATP for fatty acid synthesis.

Recently, it was reported that the cDNA-derived amino  
10 acid sequences of human ACC1 and ACC2 share 80% identity and that the most significant difference between them is in the N-terminal sequence of ACC2. The first 218 amino acids in the N-terminus of ACC2 represent a unique peptide that includes, in part, 114 of the extra 137 amino acid residues found in this isoform. Polyclonal  
15 antibodies raised against the unique ACC2 N-terminal peptide reacted specifically with ACC2 proteins derived from human, rat, and mouse tissues. These findings made it possible to establish the subcellular localization of ACC1 and ACC2 and to later demonstrate that ACC2 is associated with the mitochondria and that the  
20 hydrophobic N-terminus of the ACC2 protein plays an important role

in directing ACC2 to the mitochondria. ACC1, on the other hand, is localized to the cytosol.

Although these findings and the distinct tissue distribution of ACC1 and ACC2 suggest that ACC2 is involved in the regulation of fatty acid oxidation and that ACC1 is involved in fatty acid synthesis primarily in lipogenic tissues, they do not provide direct evidence that the products of the genes ACC1 and ACC2 have distinct roles.

These distinctions between the two ACC isoforms could not have been predicted prior to the generation of the *Acc2* knockout mouse described herein. Moreover, malonyl-CoA, the product of the ACC1 and ACC2, seems to be present in the liver and possibly in other tissues in two separate pools that do not mix and play distinct roles in the physiology and metabolism of the tissues. Malonyl-CoA, the product of ACC1, is involved in fatty acid synthesis as the donor of "C2-carbons." On the other hand, malonyl-CoA, the product of ACC2, is involved in the regulation of the carnitine palomitoyl CoA shuttle system, hence in the oxidation of fatty acids. This functional distinction between the roles of the products of ACC1 and ACC2 based on the results obtained with the *Acc2* mice was not expected nor could it have been predicted prior to this study.

Moreover, the current study demonstrates that ACC2, through its product malonyl-CoA, is potentially an important target for the regulation of obesity. Inhibition of ACC2 would reduce the production of malonyl-CoA, leading to continual fatty acid oxidation and energy production. This continual oxidation of fatty acid would be achieved at the expense of freshly synthesized fatty acids and triglycerides and of body fat accumulated in the adipose and other fatty tissues leading to reduced body fat.

The prior art is deficient in an understanding of the separate roles ACC1 and ACC2 have in the fatty acid metabolic pathways. The prior art is also deficient in assigning the differential roles of the malonyl-CoA generated by ACC1 versus that generated by ACC2 in regulating fatty acid metabolism. Also, the prior art is deficient in transgenic knockout mice generated to lack ACC2 and methods of using these transgenic mice. The present invention fulfills this long-standing need and desire in the art.

## SUMMARY OF THE INVENTION

Malonyl-CoA (Ma-CoA), generated by acetyl-CoA carboxylases ACC1 and ACC2, is the key metabolite in the regulation of fatty acid (FA) metabolism. *Acc1*<sup>-/-</sup> mutant mice were embryonically lethal, possibly due to a lack of "C<sub>2</sub>-units" for the synthesis of fatty acid needed for biomembrane synthesis. *Acc2*<sup>-/-</sup> mutant mice bred normally and had normal life spans. *Acc2*<sup>-/-</sup> mice fed normal diets did not accumulate fat in their livers as did the wild-type mice and overnight fasting resulted in a 5-fold increase in ketone bodies production, indicating higher fatty acid oxidation. ACC1 and fatty acid synthase activities and malonyl-CoA contents of the livers of the *Acc2*<sup>-/-</sup> and *Acc2*<sup>+/+</sup> mice were the same, indicating that fatty acid synthesis is unperturbed, yet the malonyl-CoA was not available for the inhibition of the mitochondrial fatty acid shuttle system, hence fatty acid oxidation was relatively high. This result was not predicted earlier to this finding, and it is very important in distinguishing the roles of the malonyl-CoA generated by ACC1 versus that generated by ACC2 in regulating fatty acid metabolism.

Absence of ACC2 resulted in 30- and 10-fold lower malonyl-CoA contents of muscles and heart, respectively. Fatty acid



oxidation in the *Acc2*<sup>-/-</sup> soleus muscles was 30% higher than that of ACC2<sup>+/+</sup> mice. Addition of insulin did not affect fatty acid oxidation in the *Acc2*<sup>-/-</sup> soleus muscle, but, as expected, it did reduce fatty acid oxidation by 50% in the wild-type soleus muscle compared to that of the mutant. This is a very important observation since it demonstrates for the first time the role of ACC2 in insulin action and regulation of fatty acid oxidation in diabetes. Isoproterenol, an analog of glucagon, had little effect on fatty acid oxidation in the muscle of the *Acc2*<sup>-/-</sup> mice but caused a 50% increase in fatty acid oxidation in the soleus muscle. Again, this result highlights the important role of ACC2 in regulating fatty acid oxidation and its potential as a target for the regulation of obesity. The higher fatty acid oxidation in the mutant mice resulted in a 50% reduction of fat storage in the adipose tissue compared to that of the wild-type mice. These results are valuable to an understanding and control of fatty acid metabolism and energy homeostasis in normal, diabetic, and obese animals, including humans.

In one embodiment of the instant invention, a method of promoting weight loss and/or fat oxidation in an individual is provided. This method may comprise the administration of an

inhibitor of acetyl-CoA carboxylase 2 (ACC2) to the individual. The same method may be used for weight loss as well.

In yet another embodiment of the instant invention, a method is provided for promoting fatty acid oxidation to treat  
5 conditions such as obesity and diabetes comprising the administration of an inhibitor of acetyl-CoA carboxylase 2 (ACC2) to an individual having such conditions.

In another embodiment of the instant invention, a method of decreasing blood sugar by administering an inhibitor of  
10 acetyl-CoA carboxylase 2 (ACC2) to an individual is provided. This method may be used to treat an individual with diabetes.

In another embodiment of the present invention, there is provided a transgenic mouse having a mutation in an endogenous gene for the ACC2 isoform of acetyl-CoA carboxylase that inactivates  
15 the protein. The ACC2 gene may be mutated by deleting one or more exons of the gene, which may be replaced by heterologous DNA sequences such as an HPRT expression cassette. In a preferred embodiment, an exon encoding a biotin-binding motif of ACC2 is replaced with an HPRT expression cassette. Unexpectedly to those in  
20 the field, these mice exhibit a phenotype consisting of a reduction in malonyl-CoA levels in skeletal muscle and heart, unrestricted fat

oxidation, and reduced fat accumulation in the liver and fat storage cells. The transgenic mice consume more food than wild-type mice but remain lean.

In yet another embodiment of the present invention,  
5 there is provided a method of screening for an inhibitor of ACC2 isoform activity consisting of the step of administering potential inhibitors to wild-type mice and screening for mice that exhibit the same phenotype of the *Acc2*<sup>-/-</sup> transgenic mice.

In yet another embodiment of the present invention,  
10 there is provided an ACC2 inhibitor identified by the above method. This inhibitor may be incorporated into a pharmaceutical composition to be administered to individuals for purposes of augmenting fatty acid oxidation and inhibiting fat accumulation to promote weight loss or maintenance.

15 The present invention has further potential for the treatment of diabetic animals, including humans, in that it may help insulin-administered type I and type II diabetics from gaining weight. Furthermore, increased fatty acid oxidation would affect carbohydrate metabolism by increasing glycolysis, and reducing  
20 gluconeogenesis and glycogen synthesis and accumulation of fatty

acid oxidation independent of insulin. Thus it helps diabetics to burn fat and lose weight.

In a further embodiment of the instant invention, a method is described for obtaining a purified preparation of ACC1 protein totally free of the ACC2 isoform by purifying ACC1 from the *Acc2*<sup>-/-</sup> transgenic mice.

In another embodiment of the instant invention, a method is provided for obtaining improved antibodies against ACC2 by generating the antibodies in the *Acc2*<sup>-/-</sup> transgenic mice.

In yet another embodiment of the instant invention, cell lines derived from the *Acc2*<sup>-/-</sup> transgenic mice are provided. Cell lines derived from muscle, heart, adipose cells, and liver cells are expected to be especially useful in bioassays and drug targeting studies. Brain cell lines including those of the hypothalamus would be useful in studying the neuropeptides involved in regulating feeding behavior and appetite and fat and carbohydrate metabolism.

In yet another embodiment of the present invention, a method of screening for agonists and antagonists of ACC2 is provided. This method comprises the steps of administering candidate compounds to *Acc2*<sup>-/-</sup> cell lines and to cell lines derived from wild-type mice followed by experiments to detect alterations in cellular

activity. A compound that specifically acts on ACC2 will alter cellular activity, fat and carbohydrate metabolism in wild-type cells but have no effect on *Acc2*<sup>-/-</sup> cells. Cellular activities that may be monitored include mRNA expression, protein expression, protein secretion, and catalytically active proteins (enzymes) involved in fatty acid and lipid and carbohydrate metabolism.

The absence of Ser 1201 in ACC2 represents an important difference between ACC1 and ACC2 regulation and can be advantageous in designing and/or generating differential inhibitor(s) [drug(s)] for ACC1 and ACC2. Other and further aspects, features, and advantages of the present invention, including the unique hydrophobic amino-terminal of ACC2, will be advantageous in designing and/or generating differential inhibitor(s) [drug(s)] for ACC1 and ACC2. Also, the differential reactions of ACC2 to anti-ACC1 antibodies would be important in designing and generating differential inhibitors for ACC1 and ACC2. Moreover, further aspects will be apparent from the following description of the embodiments of the invention given for the purpose of disclosure.

## BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others that will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate embodiments of the invention and therefore are not to be considered limiting in their scope.

**Figure 1A** shows the strategy used in the targeted mutation of the *Acc2* locus. Of the two exons (dark boxes) that were identified in the mouse genomic clone, the exon that contained the biotin-binding motif (Met-Lys-Met) was replaced with a hypoxanthine phosphorylribosyltransferase (HPRT) expression cassette to generate the targeting construct. The 3' and 5' probes used to identify the targeted events by Southern blot analysis are indicated.

**Figure 1B** shows a Southern blot analysis of the genomic DNAs extracted from mouse tails. DNA's that were digested with *Bgl*I

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were probed with the 5' probe; the DNAs digested with Bam H1 and Kpn 1 were probed with the 3' probe. DNAs from the wild-type (+/+), heterozygous (+/-), and *Acc2*-null (-/-) mice gave the expected fragment sizes.

5           **Figure 1C** shows a Northern blot of total RNA prepared from the skeletal muscles of wild-type (+/+), heterozygous (+/-), and *Acc2*-null (-/-) mice was probed with the <sup>32</sup>P-labeled 362-bp cDNA fragment, which was used to screen the genomic library. The probe detected a 10-kbp RNA band in the *Acc2*<sup>+/-</sup> and *Acc2*<sup>+/+</sup> RNAs but not  
10 in the *Acc2*<sup>-/-</sup> RNA. Hybridization of the same filter (after stripping) with a mouse β-actin cDNA probe confirmed that equal amounts of RNA were loaded in the gel.

**Figure 1D** shows a confirmation of the absence of ACC2 protein in the *Acc2*-null mice. Extracts (50 μg each) from the livers,  
15 skeletal muscles, and hearts of the mice were separated by SDS-PAGE (6%). The proteins were transferred onto a nitrocellulose filter and probed with avidin-peroxidase to detect biotin-containing proteins. The locations of the two carboxylases—the 280-kDa ACC2 and the 265-kDa ACC1—are indicated.

Figure 2 shows the relative amounts of malonyl-CoA in the tissues of wild-type (filled symbol) and *Acc2*<sup>-/-</sup> mutant (open symbol) mice. Malonyl-CoA in the acid-soluble extracts of the indicated mouse tissues was measured by the incorporation of [3H]acetyl-CoA into palmitate in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and highly purified chicken fatty acid synthase (4,29). The [3H]palmitic acid synthesized was extracted with petroleum ether and the radioactivity was measured. The mice were either fed normal chow or were fasted for 48 hours before they were sacrificed. The data are mean ± SD from three animals.

Figures 3A-3E show histological analyses of livers of 32-week-old male mice fed a standard diet. Figure 3A shows livers of wild-type (left) and *Acc2*<sup>-/-</sup> mutant mice right after 24 hours of starvation. Frozen sections of wild-type and mutant livers were stained with Oil Red-O to detect lipid droplets and counter-stained with Mayer's hematoxylin. The liver sections of wild-type mice (Figure 3B) show an abundance of red-stained lipid droplets compared to the dramatic decrease in red-stained droplets in the *Acc2*<sup>-/-</sup> mutant liver (Figure 3C). Frozen sections were made from the same livers and stained for glycogen by the periodic acid-Schiff



method and counter-stained with hematoxylin. The wild-type livers (**Figure 3D**) contain glycogen (pink-stained) and unstained lipid vacuoles, whereas the mutant livers (**Figure 3E**) have little or no glycogen and few lipid vacuoles.

5           **Figure 4** shows a summary of an experiment in which mice were sacrificed by cervical dislocation, and the soleus muscles—two from each hind limb—were resected from each mouse and were immersed in 1.5 ml of Krebs-Henseleit buffer (pH 7.4) containing 4% fatty acid-free bovine serum albumin, 10 mM glucose, and 0.3 mM [9,10(n)-<sup>3</sup>H]palmitate (3 mCi/vial) [Ibrahimi, 1999 #423]. Where indicated, insulin (10 nM) or isoproterenol (3 mM) was added, and the vials were incubated at 37°C under a humidified O<sub>2</sub>/CO<sub>2</sub> (95/5%) atmosphere for 30 min. At the end of the incubation period, the [<sup>3</sup>H]<sub>2</sub>O was separated from the labeled substrate and  
10  
15 counted.

**Figures 5A-5E** show food intake, growth (body weight) and adipose tissue in *Acc2*<sup>-/-</sup> and wild-type mice. Two groups of female mice (numbered 1 and 2; 3 and 6 weeks old, respectively) and one group of 5-week-old males—each group consisting of five  
20 *Acc2*<sup>-/-</sup> mutants (M, filled circles) and five wild type (W, open symbols)—were fed a standard diet for 27 weeks. In **Figure 5A**,

food intake was measured every week and expressed as cumulative food intake per mouse over the 27-week period. The weight of each mouse within each group was measured weekly and the data are presented as means  $\pm$  SD in **Figure 5B**. **Figure 5C** shows dorsal views of male littermates, aged 32 weeks, fed with standard diet. The amount of white fat observed under the skin of the *Acc2*<sup>-/-</sup> mouse (33.6 g weight) was much less than that of the wild-type mouse (34.2 g weight). **Figure 5D** shows an abdominal view of the fat pads under the skin of *Acc2*<sup>-/-</sup> and wild-type mice (+/+). **Figure 5E** shows epididymal fat pads isolated from the mutant (0.75 g) and wild-type (1.4 g) mice. Bar, 1 cm.

**Figures 6A** and **6B** show the targeted mutation of the *Acc1* locus. **Figure 6A** shows the strategy used to create the targeted mutation. The exon (dark box) that contains the biotin-binding motif (Met-Lys-Met) was replaced with an HPRT expression cassette. The 3' and 5' probes used for Southern blot analysis are indicated. **Figure 6B** shows a typical pattern observed in genotyping by Southern blot analyses of genomic DNA extracted from mouse tails. The DNAs were digested with *ShpI* in duplicate. The blots were probed with the 5' and 3' probes indicated in **Figure 6A**.

The presence of only wild-type (+/+) and heterozygous (+/-) genotypes indicated that no homozygous (-/-) mice were born.

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## DETAILED DESCRIPTION OF THE INVENTION

10 The instant invention is directed to a method of promoting weight loss in an individual by administering an inhibitor of acetyl-CoA carboxylase 2 (ACC2) to said individual. The same method may be used for fat reduction as well.

15 The instant invention provides a method of promoting fatty acid oxidation to treat conditions such as obesity and diabetes by administering an inhibitor of acetyl-CoA carboxylase 2 (ACC2) to an individual having such conditions.

20 The present invention provides a method of decreasing an individual's blood sugar levels by administering an inhibitor of acetyl-CoA carboxylase 2 (ACC2) to the individual. This method may be used to treat an individual with diabetes.

The present invention also provides a transgenic mouse having a mutation in an endogenous ACC2 gene for the ACC2 isoform

of acetyl-CoA carboxylase, which results in the lack of expression of a functional ACC2 isoform. This gene may be mutated by deleting one or more exons of the ACC2 gene, which may be replaced by heterologous DNA sequences such as an HPRT expression cassette.

5 Preferably, an exon encoding a biotin binding motif of ACC2 is replaced with an HPRT expression cassette. The resulting mice exhibit a phenotype consisting of a reduction in malonyl-CoA levels produced by ACC2 in skeletal muscle, heart and all other tissues, unrestricted fat oxidation, and reduced fat accumulation in the liver  
10 and fat storage cells. The transgenic mice consume more food than wild-type mice but accumulate less fat.

The present invention also demonstrates a method of screening for an inhibitor of ACC2 isoform activity consisting of administering potential inhibitors to wild-type mice and screening  
15 for mice which exhibit the phenotype of the *Acc2*<sup>-/-</sup> transgenic mice.

The present invention is also directed to an ACC2 inhibitor identified by the above method. This inhibitor may be incorporated into a pharmaceutical composition to be administered to individuals for purposes of augmenting fatty acid oxidation and  
20 inhibiting fat accumulation to promote weight loss or maintenance.

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The instant invention also provides a purification method for obtaining ACC1 protein that is free of the ACC2 isoform. This is accomplished by purifying ACC1 from tissue obtained from the *Acc2*<sup>-/-</sup> transgenic mice of the instant invention that lack the ACC2 isoform.

5 The instant invention also provides for the preparation of improved antibodies against ACC2 by generating the antibodies in the *Acc2*<sup>-/-</sup> transgenic mice. Unlike wild-type mice, these mice are less immunologically tolerant of ACC2 since it is not present during the development of immunological self-tolerance. As a result, antibodies obtained from immunization of the *Acc2*<sup>-/-</sup> transgenic mice with ACC2 are more directed to unique antigenic domains of ACC2 than similar antibodies generated in wild-type mice.

10 The instant invention is further directed to cell lines derived from the *Acc2*<sup>-/-</sup> transgenic mice. These cell lines are useful in bioassays of ACC1 and ACC2 and in drug targeting studies. Cell lines derived from the muscle, heart, adipose, and liver tissues are especially useful in these studies.

15 The instant invention also includes a method of screening for agonists and antagonists of ACC2. Candidate compounds are administered to both *Acc2*<sup>-/-</sup> cell lines and wild-type cell lines. The cells are then monitored for alterations in cellular function such as a

mRNA expression, protein expression, protein secretions, protein activities, and lipid metabolism. A compound that specifically acts on ACC2 will have altering cellular activity in wild-type cells but will have no effect on the *Acc2*<sup>-/-</sup> cell line.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

### **EXAMPLE 1**

### Generation of *Acc2*<sup>-/-</sup> Transgenic Mice

A mouse *Acc2* genomic clone was isolated using an *Acc2* cDNA probe. Based on the homology between the human and mouse ACC2 genes (Abu-Elheiga, L., Almarza-Ortega, D. B., Baldini, A., and Wakil, S.J., *J Biol Chem.* 272, 10669-10677, 1997), two oligonucleotides from the biotin-binding region based on the cDNA sequence of human ACC2 were designed: a forward primer (5'-CTGAATGATGGGGGGCTCCTGCTCT-3'; nucleotides 2551-2575) (SEQ ID No. 1) and a reverse primer (5'-TTCAGCCGGGTGGACTTTAGCAAGG-3';

nucleotides 2890-2913) (SEQ ID No. 2). These primers were used to amplify cDNA from a Quick-Clone mouse heart cDNA pool (Clontech) template.

The cDNA fragment obtained was sequenced and used to  
5 screen a 129/SvEv mouse genomic library to isolate a 16-kbp  $\lambda$  genomic clone. By digesting the 16-kbp  $\lambda$  genomic clone with different restriction enzymes, a restriction map was established and a gene targeting vector constructed that contained positive-negative selection markers and lacked the exon that contains the biotin-binding motif Met-Lys-Met (Fig. 1A). This vector was used to  
10 generate murine 129SvEv ES cells with one mutant copy of ACC2 gene (the mutant allele was termed *Acc2*<sup>tm1 LAE</sup>).

Two independent ES-cell clones were injected into mouse blastocysts, which were then implanted into the uterine horns of  
15 pseudopregnant females. Among the pups produced, eight high-level chimeras were identified and crossbred with C57BL/6J females. Each female gave birth to several agouti pups, indicating germ-line transmission of the ES-cell genome. Southern blot analysis of genomic DNA confirmed the presence of both the endogenous and the  
20 disrupted alleles in the F1 heterozygotes. The heterozygous mice were intercrossed, and their offspring were genotyped. Southern

blot analyses showed that the DNA hybridized with the 5' and 3' probes shown in Figure 1A and gave the signals expected from the wild-type (+/+), heterozygous (+/-), and homozygous-null (-/-) animals (Fig. 1B). After genotyping more than 300 mouse tails, it was determined that 24% of the progeny were *Acc2*<sup>-/-</sup>, 22% were *Acc2*<sup>+/+</sup>, and 54% were *Acc2*<sup>+/-</sup>; these results are consistent with Mendelian inheritance. The *Acc2*<sup>-/-</sup> mutants were viable, bred normally, and appeared to have a normal life span.

## EXAMPLE 2

### *Acc2* expression in *Acc2*<sup>-/-</sup> Transgenic Mice

Northern blot analyses of total RNA of skeletal muscle tissues resected from the wild-type, heterozygous, and homozygous-null animals showed no detectable *Acc2* mRNA in the homozygous-null animals and, as expected, the level of *Acc2* mRNA in the heterozygous animals was half of that in the wild-type (Fig. 1C). Western blot analyses of heart, skeletal muscle, and liver tissues from the *Acc2*<sup>-/-</sup> mutant mice using avidin peroxidase to detect



biotin-containing proteins showed no expression of ACC2 protein (Fig. 1D). The levels of ACC2 protein (280 kDa) were higher than those of ACC1 protein (265 kDa) in the heart and skeletal muscle tissues of the wild-type mice, whereas the ACC1 protein was more predominant in their liver tissues.

The absence of ACC2 protein in the *Acc2*<sup>-/-</sup> mutant mice was further confirmed by confocal immunofluorescence microscopic analysis using affinity-purified anti-ACC2-specific antibodies (Abu-Elheiga, L., W.R. Brinkley, L. Zhong, S.S. Chirala, G. Woldegiorgis, and S. Wakil. *Proc Natl Acad Sci USA.*, 97:1444-1449, 2000). Whereas the hearts, skeletal muscles, and livers of the wild-type mice had abundant expression of ACC2 antigen, there was no expression of this protein in the *Acc2*<sup>-/-</sup> mutant mice (data not shown). Thus, by all measurements, the *Acc2*<sup>-/-</sup> mutant allele is a null allele.

### EXAMPLE 3

#### Malonyl-CoA levels in *Acc2*<sup>-/-</sup> Transgenic Mice

Since the levels of malonyl-CoA in animal tissues are attributed to the activities of both ACC1 and ACC2, the consequences of the absence of ACC2 on the malonyl-CoA levels in these tissues and whether ACC1 can compensate and, consequently, raise the levels of malonyl-CoA in these tissues was determined. In comparing the liver tissues of the wild-type and *Acc2*<sup>-/-</sup> mutant mice, there were no significant differences in the malonyl-CoA levels and overall ACC activities, suggesting that almost all of the malonyl-CoA in the liver is contributed by ACC1 (Fig. 2).

On the other hand, in comparing the skeletal muscle and heart tissues of the same two groups of mice, the levels of malonyl-CoA was found to be about 30- and 10-fold lower, respectively, in these tissues of the *Acc2*<sup>-/-</sup> mutant mice than in those of the wild-type mice. This suggests that ACC2 is the main contributor of malonyl-CoA in skeletal muscle and heart (Fig. 2).

During fasting, the levels of malonyl-CoA dropped comparably in the liver tissues of both the wild-type and the *Acc2*<sup>-/-</sup> mutant mice, suggesting that ACC1 is affected by dietary conditions



the same for both groups. However, the livers of wild-type mice were lighter in color than the mutant livers, suggesting that they contained more fat (Figure 3A).

To confirm this supposition, liver tissues were stained with Oil Red-O to detect lipids and estimate their lipid and triglyceride contents. Wild-type livers contained abundant lipid droplets (Fig 3B), which are primarily triglycerides, whereas *Acc2*<sup>-/-</sup> livers contained significantly fewer lipid droplets (Fig. 3C). Extraction and analysis of the total lipids by thin-layer chromatography showed that the mutant livers contained 20% less lipid than wild-type livers, and the triglyceride content of the lipid was 80% to 90% lower than wild-type.

## **EXAMPLE 5**

### **ACC1 and ACC2 modulate distinct pools of malonyl CoA.**

Since the activities of ACC and fatty acid synthase (FAS) activities in liver extracts of wild-type and *Acc2*<sup>-/-</sup> mutants were the same, the difference in the liver lipid content must be secondary to uncontrolled mitochondrial fatty acid oxidation in the *Acc2*<sup>-/-</sup> livers

rather than due to a suppression of fatty acid synthesis. Also, because malonyl CoA is a negative regulator of the mitochondrial carnitine palmitoyl-CoA shuttle system (McGarry, J.D., and N.F. Brown., *Eur. J. Biochem.*, 244:1-14, 1997), its absence in *Acc2*<sup>-/-</sup> livers would be expected to increase fatty acid translocation across the mitochondrial membrane and subsequent  $\beta$ -oxidation. Thus, these results suggest that malonyl-CoA, synthesized by ACC2, affects the accumulation of fat in the liver by controlling fatty acid oxidation. Since ACC1-generated malonyl-CoA, which is abundant in the livers of both groups of mice, apparently did not inhibit the  $\beta$ -oxidation of fatty acids, it can be concluded that the malonyl-CoA produced by ACC1 and ACC2 exists in two distinct compartments of the cell—the cytosol and the mitochondria, respectively, and carries out distinct functions in these compartments. Because both ACC1 and ACC2 are present in both the periportal (zone 1) and perivenous (zone 3) hepatocytes of rat liver, it is unlikely that the two pools of malonyl-CoA were derived from differential expression of ACC1 and ACC2 in these discrete regions of the liver.

## EXAMPLE 6

### Analysis of glycogen in the liver of *Acc2*<sup>-</sup> transgenic mice

Glycogen, the storage form of glucose in the liver and muscles is an important regulator of energy homeostasis in animals including humans. Its synthesis and degradation is closely related to glucose metabolism. The enzymes involved in glycogen metabolism are highly regulated by hormones such as insulin, glucagon, and epinephrine.

To examine whether the loss of ACC2 affects the level of glycogen, frozen sections of livers resected from wild-type and *Acc2*<sup>-/-</sup> mutant mice were stained for glycogen (Figures 3D and 3E). In the nourished state, the wild-type livers contained abundant amounts of glycogen (410±10 µmol/g of wet tissue), whereas the livers of *Acc2*<sup>-/-</sup> mice contained 20% less glycogen (325± 14 µmol/g of wet tissue).

Speculation suggests that more glucose is utilized in the synthesis of fatty acids and their subsequent oxidation in the *Acc2*<sup>-/-</sup> liver, thus depleting glycogen. In the 24-hour-fasted wild-type mouse liver, glycogen is clearly present (Figure 3D), whereas it was undetectable in the *Acc2*<sup>-/-</sup> mutant liver (Figure 3E).

## EXAMPLE 7

### Analysis of blood glucose and lipids in *Acc2*<sup>-/-</sup> transgenic mice

5           The next step was analysis of the serum levels of cholesterol, glucose, triglycerides, free fatty acids and ketone bodies in wild-type and *Acc2*<sup>-/-</sup> mice fed a standard diet. Cholesterol levels were similar in both groups of mice ( $92.8 \pm 3.1$  and  $95.1 \pm 7.4$  mg/dl), and glucose levels were 20% lower in mutant mice ( $176.6 \pm 6.5$  versus  $136.2 \pm 5.4$  mg/dl). Fatty acid levels were lower in mutant mice ( $1.37 \pm 0.31$  versus  $0.84 \pm 0.12$  mM), whereas triglyceride levels were 30% higher in the mutant mice ( $35.1 \pm 2.5$  versus  $45.2 \pm 5.9$  mg/dl), possibly due to mobilization of triglycerides and fatty acids from liver and/or adipose for their delivery to the heart and muscles as a substrate for oxidation. Serum levels of the ketone bodies ( $\beta$ -hydroxybutyrate) were nearly undetectable in both the wild-type and mutant mice. However, an overnight fast (10 to 12 hours) increased the blood  $\beta$ -hydroxybutyrate concentration of the *Acc2*<sup>-/-</sup> mice fourfold over that of the wild type ( $2.5 \pm 0.6$  mM versus  $0.7 \pm 0.05$  mM, n=5),

consistent with a higher degree of fatty acid oxidation in the mutant mice.

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### EXAMPLE 8

#### Fatty Acid Oxidation in *Acc2*<sup>-/-</sup> transgenic mice

To provide further evidence for the role of ACC2-synthesized malonyl-CoA as the regulator of fatty acid oxidation, fatty acid oxidation was investigated in the mouse soleus muscle, a type II muscle tissue responsive to hormonal regulation (Vavvas, D., Apazidis, A., Saha, A.K., Gamble, J., Patel, A., Kemp, B.E., Witters, L.A., and Ruderman, W.B., *J Biol Chem.*, 272:13255-13261 1997; Alam, N., and E.D. Saggerson. *Biochem J.*, 334:233-41, 1998;; Abu-Elheiga, L., Jayakumar, A., Baldini, A., Chirala, S.S., and Wakil, S.J. *Proc Natl Acad Sci. USA* 92, 4011-4015, 1995; Abu-Elheiga, L., Almarza-Ortega, D. B., Baldini, A., and Wakil, S.J. *J Biol Chem.* 272, 10669-10677, 1997; - Ha, J., J.K. Lee, K.-S. Kim, L.A. Witters, and K.-H. Him. *Proc Natl Acad Sci USA.* 93:11466-11470, 1996; Rasmussen, B. B. and Wolfe, R.R., *Ann. Rev. Nutr.* 19:463, 1999; and, Bressler, R. and Wakil, S.J. *J Biol Chem.* 236:1643-1651, 1961).



As shown in Figure 4, the oxidation of [<sup>3</sup>H]palmitate was 30% higher in the isolated soleus muscles of *Acc2*<sup>-/-</sup> mutant mice than in those of the *Acc2*<sup>+/+</sup> mice. Insulin is known to activate both ACC1 and ACC2 and, thereby, to induce fatty acid synthesis and inhibit fatty acid oxidation, respectively. Adding insulin to soleus muscles resected from wild-type and from *Acc2*<sup>-/-</sup> mutant mice did not affect fatty acid oxidation in the *Acc2*<sup>-/-</sup> mutant muscle cells (Fig. 4) but did reduce palmitate oxidation by about 45% in the wild-type muscle cells (Fig. 4). Based on these results, it can be concluded that the insulin-mediated inhibition of β-oxidation occurs through the activation of ACC2, probably by dephosphorylation (Lopaschuk, G., and Gamble, J. *Can J Physiol Pharmacol.* 72:1101-1109. 1994; Kudo, N., Bar, A.J., R.L., Desai, S., Lopaschuk, G.D. *J Biol Chem.* 270:17513-17520, 1995; Dyck, J.R., N. Kudo, A.J. Barr, S.P. Davies, D.G. Hardie, and G.D. Lopaschuk. *Eur J Biochem.* 262:184-190, 1999; Vavvas, D., Apazidis, A., Saha, A.K., Gamble, J., Patel, A., Kemp, B.E., Witters, L.A., and Ruderman, W.B. *J Biol Chem.* 272:13255-13261, 1997; Iverson, A.J., A. Bianchi, A.C. Nordlund, and L.A. Witters. *Biochem J.* 269:365-371, 1990; Kim, K.H., F. Lopez-Casillas, D.H. Bai, X. Luo, and M.E. Pape. *Faseb J.* 3:2250-2256, 1989; Thampy, K.G., and Wakil, S.J. *J. Biol.*

Chem. 263, 6454-6458, 1988; Mabrouk, G.M., Helmy, I. M., Thampy, K.G., and Wakil, S.J. *J. Biol. Chem.* 265, 6330-6338, 1990; Mohamed, A.H., W.Y. Huang, W. Huang, K.V. Venkatachalam, and S.J. Wakil. *J Biol Chem.* 269:6859-6865. 1994; and, Hardie, D.G. *Prog Lipid Res.* 28:117-146, 1989).

The role of ACC2 in the regulation of mitochondrial oxidation of fatty acids was further confirmed by using isoproterenol, an analog of glucagon, which produces effects opposite of those of insulin. Adding isoproterenol to wild-type soleus muscle increased palmitate oxidation by 50% (Fig. 4), raising it to nearly the same level as that found in the mutant muscle cells. It is noteworthy that isoproterenol also further increased fatty acid oxidation in the mutant soleus muscle cells (Fig. 4). This additional increase may be due to factors independent of malonyl-CoA (Kim, K.H., F. Lopez-Casillas, D.H. Bai, X. Luo, and M.E. Pape. *Faseb J.* 3:2250-2256, 1989).

Altogether, these results confirm for the first time that mitochondria-associated ACC2, and not cytosolic ACC1, is responsible for the insulin-mediated activation and isoproterenol (glucagon)-mediated inactivation that results, respectively, in decreased and increased fatty acid oxidation. Since the mitochondrial CPTI activities of the soleus muscles from both groups of mice were very

similar (data not shown), the observed effects of these hormones are solely due to their effect on ACC2.

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## **EXAMPLE 9**

### **Feeding experiments with *Acc2*<sup>-/-</sup> transgenic Mice**

It appears that the mitochondrial  $\beta$ -oxidation of fatty acids occurs in the *Acc2*<sup>-/-</sup> mutant mice in an unregulated yet sustained manner. To investigate the role of this type of fatty acid  $\beta$ -oxidation and its effect on food consumption and weight gain, feeding experiments were performed with three groups of mice (each group consisting of 5 wild-type and 5 *Acc2*<sup>-/-</sup> mutant mice) that were fed a weighed standard diet *ad liberatum*. (Fig. 5 represents a plot of one of the groups). Food consumption (no spillage was noted) for each group was measured every week for 27 weeks, and the weight of each mouse was recorded weekly.

On the average, each *Acc2*<sup>-/-</sup> mutant mouse consumed 20-30% more food per week than the wild-type mice (Figure 5A) and

maintained an average body weight of 21 g compared to 23 g per wild-type mouse. The *Acc2*<sup>-/-</sup> mutant mice were generally leaner, weighing about 10% less than the wild-type mice throughout the feeding periods (Figure 5B). In addition, *Acc2*<sup>-/-</sup> mutant mice accumulated less fat in their adipose tissues (Figures 5C and 5D). For example, the epididymal fat pad tissue in an *Acc2*<sup>-/-</sup> male weighed 0.75 g as compared to 1.4 g in a wild-type male littermate (Figure 5E). The decrease in the adipose size resulted in a decrease in the leptin released to the plasma from 53 ± 9 ng/ml in the wild-type mice to 36 ± 3 ng/ml in the mutant mice. Thus, mitochondrial β-oxidation of fatty acids regulates fat storage in the adipose tissue.

### EXAMPLE 10

#### Generation of *Acc1*<sup>-/-</sup> transgenic mice

To demonstrate the importance of ACC1 in the *de novo* synthesis of fatty acids, the same strategy was followed to generate an *Acc1*-knockout mouse as done for *Acc2*. Like ACC2, the ACC1 isoform is also highly conserved among animal species (Thampy, K.G.

5 *J Biol Chem.* 264:17631-17634, 1989). A forward primer (5'-  
GGATATCGCATCACAATTGGC-3') (SEQ ID No. 3) based on the human  
*Acc1* cDNA and a reverse primer (CCTCGGAGTGCCGTGCTCTGGATC-3')  
(SEQ ID No. 4) that contained the biotin-binding site was designed  
and used to amplify a 335-bp cDNA probe using human cDNA as a  
template. A 129/SvEv mouse genomic library was screened with the  
PCR fragment as described for ACC2, and a 14-kbp clone was  
isolated, mapped with restriction enzymes, and analyzed by Southern  
blotting (Fig. 6B). A correctly targeted clone (Fig. 6A) was  
10 microinjected into C57BL/6J mouse blastocysts, which were then  
implanted into the uterine horns of pseudopregnant female mice.  
The male chimeras thus generated were bred with C57BL/6J mates,  
and the *Acc1* heterozygous offspring were interbred.

After analyzing genomic DNA from more than 300  
15 progenies by Southern blotting using both the 5' and 3' probes,  
homozygous *Acc1*-null mutant offspring were not obtained. The  
litter sizes were less than average, being 6 or 7, and 35% of the  
progeny were wild-type and 65% were heterozygous. These results  
demonstrate that the *Acc1* mutation is embryonically lethal.

20 To characterize this embryonic lethality, the mating of  
the heterozygotes was timed and the resulting embryos were

genotyped. At gestation days E12.5 and E13.5, the viable embryos were 35% wild-type and 65% heterozygous, indicating that the lethality had occurred earlier. At gestation day E9.5, the remains of dead embryos were recovered, and at gestation day E8.5, 5 degenerating embryos were recovered from inside the ectoplacental cone.

### Discussion

Obesity is a major health factor that affects the body's susceptibility to a variety of diseases such as heart attack, stroke, and diabetes. Obesity is a measure of the fat deposited in the adipose in response to food intake, fatty acid and triglyceride synthesis, fatty acid oxidation, and energy consumption. Excess food provides not only the timely energy needs of the body, but promotes glycogen synthesis and storage in liver and muscle and fatty acid and triglyceride synthesis and storage in the fat tissues. Calorie restriction or starvation promotes glycogenolysis that supplies glucose where needed and lipolysis that supplies fatty acids for oxidation and energy production. Insulin and glucagon are the hormones that coordinate these processes. Malonyl-CoA is the key intermediate in fatty acid synthesis and has recently assumed an

additional role as a second messenger that regulates energy levels (ATP) through fatty acid oxidation, which in turn affects fatty acid synthesis and carbohydrate metabolism.

The studies described above provide a definitive  
5 characterization of the role of malonyl-CoA produced by ACC2 in the regulation of fatty acid oxidation and energy metabolism. Malonyl-CoA generated by ACC1 is the donor of the C<sub>2</sub> units required for fatty acid synthesis. Acetyl CoA, the substrate for ACC1 and ACC2, is the product of pyruvate oxidation, hence studies of the carboxylases  
10 interrelate three major metabolic pathways—carbohydrate metabolism, fatty acid synthesis, and fatty acid oxidation.

Studies on animal carboxylases, usually a mixture of ACC1 and ACC2, showed that these enzymes are under long-term control at the transcriptional and translational levels and under  
15 short-term regulation by phosphorylation/dephosphorylation of targeted Ser residues and by allosteric modifications by citrate or palmitoyl-CoA. Several kinases have been found to phosphorylate both carboxylases and to reduce their activities. Insulin activates the carboxylases through their dephosphorylation, whereas glucagon and  
20 epinephrine inactivate them as a result of their phosphorylation. The AMP-activated protein kinase (AMPK), one of the most notable

kinases, is activated by a high level of AMP concurrent to a low level of ATP through mechanisms involving allosteric regulation and phosphorylation by protein kinase (AMPK kinase) in a cascade that is activated by cellular stressors that deplete ATP. Through these mechanisms, when metabolic fuel is low and ATP is needed, both the ACC activities are turned off by phosphorylation, resulting in the low malonyl-CoA levels that lead to increased synthesis of ATP through increased fatty acid oxidation and decreased consumption of ATP for fatty acid synthesis.

The differential expression of ACC1 and ACC2 in various tissues—ACC1 is highly expressed in liver and adipose and ACC2 is predominant in heart and muscle—and their cellular localization—ACC1 in the cytosol and ACC2 on the mitochondrial membrane—suggest that their functions are different though interrelated. The cytosolic ACC1-generated malonyl-CoA is utilized by the fatty acid synthase, which also is a cytosolic enzyme, for the synthesis of fatty acids. The mitochondrial ACC2-generated malonyl-CoA functions as a regulator of CPTI activity—CPTI being the first enzyme that catalyzes the shuttling of long-chain fatty acids into the mitochondria for  $\beta$ -oxidation and energy production. ACC2-



generated malonyl-CoA, therefore, is a second messenger that regulates ATP levels through fatty acid oxidation, which, in turn, affects fatty acid synthesis and carbohydrate metabolism.

The present studies of the *Acc2* mutant mice strongly support this conclusion. The levels of malonyl-CoA in the livers of the mutant mice were similar to those in the livers of the wild-type mice, indicating its synthesis by ACC1, the predominant carboxylase in this tissue. In the livers of the wild-type mice, the malonyl-CoA is used to synthesize fatty acids, which are then converted into triglycerides that accumulate as lipid droplets (Fig 3A). In the livers of the *Acc2*<sup>-/-</sup> mutant mice, the uncontrolled CPTI activity results in the oxidation of fatty acids by the liver mitochondria or in the conversion of fatty acids into lipids (very-low-density lipoproteins), which are then transported through the bloodstream to the heart and muscles to overcome the increased demand of these tissues for fatty acids consequential to uninhibited CPTI activity and amplified fatty acid oxidation. These conclusions were supported by the near absence of malonyl-CoA in the heart and skeletal muscle tissues of the *Acc2*<sup>-/-</sup> mutant mice, by the higher fatty acid-oxidation rate in the soleus muscles of the *Acc2*<sup>-/-</sup> mutant mice, and by the occurrence

of fatty acid oxidation independent of insulin and isoproterenol, an analog of glucagon (Fig. 4).

Finally, knocking out ACC2 in mice has demonstrated that the lack of malonyl-CoA, the mitochondrial second messenger, produces offspring that exhibit increased oxidation of fatty acids, decreased accumulation of lipids, and decreased storage of glycogen in the liver but are still morphologically normal, grow at an expected rate, and breed normally (their longevity and aging are being followed). All of the metabolic changes are expressed in food consumption patterns and body weight—the *Acc2*<sup>-/-</sup> mutant mice who were fed a standard diet typically consumed 20% more food than did the wild-type mice yet eventually lost 10% of their body weight.

The reduction in fat content and the size of the adipose tissue led to a reduction of about 30% in leptin released to the plasma, similar to that occurring in fasted mice. This signaled the hypothalamus to produce the appetite-stimulating neuropeptide Y, which promotes feeding. This is the most plausible explanation for the observation that *Acc2*<sup>-/-</sup> mice have smaller fat stores even as they consumed more food than the wild-type mice (Figures 5A-5E). It has been suggested that malonyl-CoA may play a role in signaling the

availability of physiological fuel by acting through the hypothalamic neurons. This suggestion was based on the inhibition of ACC by 5-(tetradeculoxy)-2 furoic acid that increases food uptake in mice treated with fatty acid synthase inhibitors. Although this possibility  
5 could not be ruled out in the *Acc2*<sup>-/-</sup> mice, the lower leptin levels in the plasma may be sufficient to increase appetite. Moreover, the *Acc2*<sup>-/-</sup> mice appear to be normal, with no obvious neurological abnormalities.

Maintenance of high levels of fatty acid oxidation results  
10 in reduced fat accumulation and storage, a physiological state that humans try to attain through exercise. Pharmacological inhibition of ACC2 may allow individuals to lose weight while maintaining normal caloric intake.